tags, and the application of at least two different affinity purification steps for detecting and/or purifying the biomolecules or their complexes, respectively (cf. claims 1 and [illegible]). Surprisingly, the inventive method enables the generation of highly purified biomolecules or biomolecule complexes, which exhibit their natural activity or are present in a form of their natural complexes, respectively.

Expression in natural environment and purification of thus obtained proteins up to homogeneity is in contrast to the methods previously used in the prior art. Therein, a protein is usually over-expressed in a foreign host (cf. page 1, third paragraph to page 2, first paragraph of the specification). The over-expressed protein is often present in so-called inclusion bodies, which have to be resolubilized before further treatment. While such methods using over-expression work well for conventional protein detection methods based on weight analysis (e.g. polyacrylamide gels, Western blots, etc.) they are not particular, not for assays on protein complexes. In contrast thereto, according to the invention, an expression environment is set up which facilitates expression of protein in native form, which includes that the transcriptional control sequences are preferably selected so that the fusion protein is not overexpressed, but expressed at basal levels in the cell (cf. page 7, first paragraph). Further, to express the protein in a native form, as defined on page 7, first paragraph of the specification, the protein or protein complex to be purified is preferably expressed in its natural host (cf. page 6, lines 21-[illegible]).

Since the purification method according to the invention is very efficient, it is not necessary to over-express the proteins to be purified, but the proteins can be expressed at their basal levels. This has the advantage of avoiding the formation of inclusion bodies and also reduces the risk of toxic effects in the cell that have amounts of protein [illegible]. Furthermore, this allows the purification

of proteins of interest in their natural occurring complex situation.

According to the invention, the protein or protein complexes being expressed in a native form as fusion proteins includes at least two different affinity tags are subjected to a very efficient and at same time very gentle purification using two affinity steps. The use of high affinity tags, namely IgG binding domains of Staphylococcus protein A allows for purification of protein expressed at a low level. A second affinity purification step then surprisingly results in pure proteins, being freed of remaining contaminants and of optionally used proteases.

Thus, the system according to the invention allows for sufficient purification of proteins expressed at low level in native form, preferably in their natural hosts, while maintaining them in functional complexes. It was neither known nor obvious from the state of the art that a combination of two affinity tags could be used for this purpose.

<u>Rejections under 35 USC 102</u>

Darzins et al. (WO 96/41943) relates to a method for expressing a desired protein in gram-positive bacteria. The method described in Darzins et al. is a process completely different to the inventive method.

Darzins et al. neither describes the expression of proteins in native form nor the use of at least two different affinity purification steps for detecting and/or purifying biomolecules or biomolecule complexes.

Darzins et al. rather relates to a very specific method for the expression of proteins in gram-positive bacteria, i.e. <u>not</u> in their natural host. In contrast to the present invention, wherein a polypeptide fused to two affinity tags is expressed, according to Darzins et al. a fusion protein is expressed comprising an N-terminal signal sequence and a C-terminal sorting sequence. It is pointed out that these

sequences [illegible] according to Darzins et al. are not affinity tags, but sequences which are necessary so that the protein desired according to Darzins et al. is either anchored at the cell surface or secreted into the extra-cellular space, i.e. the fused sequences are required for successful translocation (cf. page 13, line 15 to page 14, line 5 of Darzins et al.). Accordingly, a construct suitable according to Darzins et al. e.g. contains 122 N-terminal and 140 C-terminal amino acids of M6 protein.

Further, in Darzins et al. the protease is used for a totally different purpose than in the inventive method (optionally there). The protease according to Darzins et al. serves to cleave off the C-terminal sequence (anchor sequence), with which the expressed fusion protein is bound to the host cell surface. Neither binding to the host cell surface, nor cleavage thereof are intended by the inventive method.

According to the invention it is essential that the protein is expressed in <u>native form</u> as fusion protein and then can be purified with the help of the inventive purification method in two affinity purification steps despite low abundance expression. Contrary thereto, the method according to Darzins et al. concerns a very specific expression of proteins at certain sites of gram-positive bacteria with the help of N-terminal signal sequences and C-terminal sorting sequences (which are not necessary according to the invention), wherein the desired protein is <u>overproduced</u>, as usually done in the state of the art prior to the present invention (cf. e.g. page 14, lines 6-7). This overproduced protein is then purified according to Darzins et al., optionally after cleavage of the anchor sequence to be released from the cell surface, when only methods known in the art such as single-step purification using [illegible]

Staphylococcus protein A or on the fact that these proteins expressed in native form can be purified due to the high efficiency of the method.

The method according to the invention is thus not anticipated by the completely different method according to Darzins et al.

Further differences between the method according to the invention and the method according to Darzins et al. and the advantages resulting thereof are explained as follows:

Darzins et al. only describes purification of proteins from gram-positive bacteria. The present patent application, however, can be applied to any organism in which recombinant DNA can be introduced. In fact, as indicated in the application, expression in a natural host is possible and even favored (page 6, line 20). Darzins et al. cannot be used to purify protein from organisms (e.g. eucaryotes) assembled in natural complexes and/or carrying the correct post-traductional modifications often required for biological activity.

The main application of Darzins et al. is to overproduce a single recombinant protein in a heterologous environment. The main application of the system described in the present application is the purification of protein complexes in a natural environment, including when complex composition is not known in native form. Darzins et al. is not suited for protein complex purification and cannot be used at all when complex composition is unknown. The goal of Darzins et al is to produce a large amount of a single protein (cf. Field of the Invention of Patent WO 96/40943).

Since the goal of Darzins et al. is to produce a large amount of a single protein (cf. Field of the Invention of Patent WO 96/40943), the protein is over-expressed according to Darzins et al. In these conditions, only low affinity purification methods are required to obtain pure protein from [illegible]. The strategy is specifically designed [illegible]

recover minute amounts of protein expressed at their normal cellular level, providing the best condition for post-translational modification and association with previously unknown partners. Darzins et al. is not suited for the recovery of minute amounts of protein expressed in their natural environment.

The method described in Darzins et al. requires the fusion of an N-terminal signal sequence to the target protein. The target protein, fused to these sequences, has further to be competent to be exported to extra-cellular domain of a gram-positive bacterium, which requirement may rise problems (e.g. large heterologous protein, hydrophobic protein, . . .) Furthermore, some proteins may not tolerate fusions at their N-terminus for expression and folding. In our system fusion of affinity tags can be done at either of the protein extremities or at both. Furthermore, the protein can be expressed in various cellular compartments (not obligatorily secreted), with a strong preference for its natural host compartment. Darzins et al. provides some constraints on protein production and a single specific environment that severely restricts its use.

Proteins produced as described in Darzins et al. can be purified in two steps only if they are first anchored to the host cell surface for the first purification step. A second optional purification step can be performed after release of the protein from the protein form the cell surface. These conditions (e.g. binding to the cell surface) are not suitable to purify protein and interacting partners that must be assembled in specific conditions (e.g. cellular environment of a eukaryotic host at a specific time point of the cell cycle). Using our method, the target protein is preferentially in its natural host and assembles naturally with it's partners. The target protein can be expressed in a soluble form to allow assembly and modification. This allows purification of naturally assembled complexes, even if very specific assembly conditions are required. Multi-step purification performed as

described in Darzins et al. necessitates production of the protein of a membrane attached form preventing its use for many applications.

According to the present invention, one or several IgG binding domains of Staphylococcal protein A are used and not the signal and anchor sequences of this protein as described in Darzins et al. The main tags used in Darzins et al. and the present application are therefore different.

The present strategy is also novel because it allows skilled persons to purify proteins in a way that cannot be achieved by using the method described in Darzins et al.

Rejections under 35 USC 103

The subject matter of the present invention is not rendered obvious by combining the cited Darzins et al. and Zheng et al. either.

Zheng et al. (Gene 186 (1997, 55-60)) only reports the use of calmodulin-binding protein for the purification of proteins over-expressed in E.coli (cf. e.g. page 55, first paragraph under the title "Introduction", page 56, right column under 2.1 as well as page 60, left column, lines 11-12). Further, Zheng does not suggest a purification procedure using at least two different affinity purification steps but rather the use of a single step using calmodulin affinity chromatography (cf. abstract, lines 2-3).

The present invention therefore provides a system allowing efficient purification of proteins expressed at low level, preferably in their natural hosts, while maintaining them in functional complexes. It was not known previously that a combination of two affinity tags could be used for this purpose. The combination of tags required for this new application was not known and previously publications did not permit that the combination disclosed would be successful.

Moreover, the invention strategy surprisingly enables purification of proteins expressed at their natural

level. This will ease the detection of interacting partners and represents an unexpected advantage.

Requirements under 35 USC 112

Claim 1 has been amended to further clarify the term "subunits" by indicating "subunits of biomolecule complexes". Support for this amendment is at page 5, line 20 to page 6, line 2 of the specification.

Claim 5 has been amended to spell out a definition of "TEV". "NIA" denotes a protease type and is not an abbreviation. The TEV protease is described at page 9, line 16 of the specification.

Conclusion

In view of the amendments and remarks made herein, applicant respectfully requests that the application be passed to issue.

The Commissioner is hereby authorized to charge any additional fees which may be required in this application to Deposit Account No. 06-1135.

Respectfully submitted,
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By
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Date: [illegible]

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<u>Version with Markings to Show Changes Made</u>

1. (Once amended) Method for detecting and/or purifying substances selected from proteins, biomolecules, complexes of proteins or biomolecules, subunits <u>of biomolecule complexes</u> [thereof], cell components, cell organelles and cells comprising the steps;

(a) providing an expression environment containing one or more heterologous nucleic acids encoding one or more polypeptides and/or one or more subunits of a biomolecule complex, the polypeptides or subunits being fused to at least two different affinity tags, one of which consists of one or more IgG binding domains of Staphylococcus protein A,

(b) maintaining the expression environment under conditions that facilitate expression of the one or more polypeptides or subunits in a native form as fusion proteins with the affinity tags,

(c) detecting and/or purifying the one or more polypeptides or subunits by a combination of at least two different affinity purification steps each comprising binding the one or more polypeptides or subunits via one affinity tag to a support material capable of selectively binding one of the affinity tags and separating the one or more polypeptides or subunits from the support material after substances not bound to the support material have been removed.

[illegible]. (Once amended) Method according to claim 4, wherein the specific proteolytic cleavage site is the cleavage site for <u>Tobacco Etch Virus</u> [TEV] protease NIA.